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In Vitro Measurements of Cytotoxic Effects of 193 nm and 213 nm Laser Pulses at Subablative Fluences

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Background and Objective: **The frequency-quintupled q-switched Nd:YAG laser is being studied as an alternative to the ArF excimer laser for photorefractive procedures. The present report describes two experiments comparing biologic effects of these laser devices.**

Study Design/Materials and Methods: **Bovine corneas were irradiated with subablative laser pulses in liquid nitrogen and analyzed by electron paramagnetic resonance spectroscopy to assess free radical production. Aqueous bacterial suspensions were irradiated with low-intensity laser pulses and assayed for cell survival.**

Results: **Electron paramagnetic resonance spectra were very similar in both amplitude and shape for exposure at the two wavelengths. Bacterial survival was markedly less for 213 nm irradiation than 193 nm exposure and displayed a different dependence on cumulative exposure.**

Conclusion: **Free radical production by 213 nm laser exposure is quite comparable to that seen previously for 193 nm irradiation. However, cell lethality appears to be significantly greater at the longer ultraviolet wavelength. This difference may contribute to complications observed after corneal photoablation with the 213 nm device. Lasers Surg. Med. 21:88–93, 1997.**

Key words: cytotoxicity; free-radicals; corneal ablation; photorefractive keratectomy

INTRODUCTION

Photorefractive keratectomy (PRK) is based on the fact that intense pulsed radiation in the 200 nm wavelength range ablates cornea with submicron precision and minimal damage to the remaining tissue [1]. Nearly all PRK studies to date have used as the UV source the argon fluoride (ArF) excimer laser, which has a wavelength of 193 nm and a pulse duration of ~ 10 ns [2]. However, the physical dimensions, often poor initial beam homogeneity, and gas-handling requirements of the ArF laser have prompted several efforts to find a solid-state laser alternative. Toward this end, particular attention has been given to the frequency-quintupled q-switched neodymium: yttrium aluminum garnet (Nd:YAG) laser, which produces 5 to 7 ns pulses at 213 nm.

In vitro studies of the laser-tissue interaction for 213 nm radiation have found corneal ablation rates similar to those obtained at 193 nm (i.e., within approximately a factor or two for comparable pulse fluences), as well as minimal bulk tissue damage [3–7]. Although once quintupled the Nd:YAG laser energy per pulse is much lower than that of the ArF device, i.e., a few millijoules or less, by focusing the 213 nm beam and scanning it across the treatment zone at a high pulse rate comparable corneal reshaping is theoretically possible.

Recent in vivo work in rabbits provides less

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clear evidence as to the appropriateness of the Nd:YAG laser for PRK. In one study the tissue response was comparable for 193 nm and 213 nm procedures [8], but in another the results were significantly worse at the longer wavelength [9]. Specifically, epithelial healing, corneal vessel ingrowth, and accuracy of refractive change were found to be significantly worse for the solid-state device than typical outcomes with excimer laser treatment. Unclear is whether these adverse tissue healing effects were due to the small spot scanning method employed by that particular Nd:YAG laser system or the 213 nm laser wavelength.

Here, we report two experiments comparing measurable biologic effects of 213 nm and 193 nm laser radiation. First, we examine free radical formation in cornea using electron paramagnetic resonance spectroscopy (EPR). Previously we performed a similar experiment to show ArF laser generation of radicals in the tissue, with a quantum efficiency of ~ 10^{-3} radicals per 193 nm photon (at ablative laser fluences) [10]. Second, we use a bacterial cell survival assay to assess the relative cytotoxicity of the two laser wavelengths. In both experiments the laser spot size and repetition rate are kept the same for the ArF and Nd:YAG lasers.

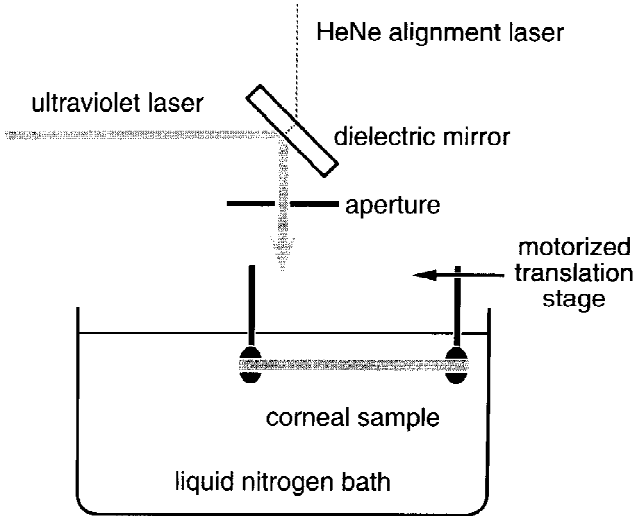

Fig. 1. Schematic of the experimental apparatus for ultraviolet laser exposure of bovine corneal samples prior to EPR spectroscopy. A HeNe laser coaxially aligned with the UV beam aided alignment of the sample prior to exposure. The motorized translation stage scanned the sample at ~ 0.5 mm/sec over a range of 20 mm. Tissue samples were immersed in liquid nitrogen during exposure and remained at cryogenic temperatures throughout all phases of the experiment.

MATERIALS AND METHODS

Free Radical Formation

Tissue samples used in this work were bovine corneas. Whole globes were obtained from a slaughterhouse and stored on ice until use < 6 hours postmortem. The following tissue handling steps were designed to preserve the physiologic hydration state of the tissue as much as possible. Immediately prior to laser irradiation, the central cornea of each globe was mechanically de-epithelialized and cut, by a die containing two parallel razor blades, into rectangular strips measuring ~ 25 mm × 2 mm. Each strip was placed lengthwise on a glass stirring rod with the anterior stromal surface oriented upward and quickly immersed in liquid nitrogen (LN_2). This preserved the rectangular sample shape, and the slight curvature induced by the underlying rod helped identify the anterior sample surface, i.e., the corneal surface treated in clinical photorefractive procedures.

The irradiation apparatus is shown in Figure 1. The collimated, spacially uniform output from either an ArF excimer laser (193 nm) or a quintupled Nd:YAG laser (213 nm) was reflected by a dielectric mirror downward into an LN_2 bath containing the corneal tissue sample. Irradiation in liquid nitrogen was required because the radical species are highly unstable at room temperature [10]. A HeNe laser coaxial with the ultraviolet (UV) laser beam was used for alignment. Each laser pulse delivered 1.5 mJ through a 3.25 mm diameter aperture (18 mJ.cm^{-2}) at a repetition rate of 2 Hz. The liquid nitrogen reduced the laser fluence reaching the immersed tissue by an unknown amount, although a cursory examination (i.e., ablation of a polyimide target either above or below the LN_2 surface) suggested the radiation loss was small. Consequently, from geometrical considerations (i.e., beam size, fluence, and corneal target width), each laser pulse delivered ~ 1 mJ to the sample. For reference, the ablation threshold fluence of bovine cornea at 193 nm—at room temperature—is ~ 30 mJ.cm^{-2} [11], and the fluences used in most human PRK studies are in the 150–200 mJ.cm^{-2} range. Thus the fluence used in this work was subablative.

The corneal tissue strips were mounted on a horizontal brace just beneath the LN_2 surface level. The brace was fixed to a motorized translation stage that repetitively scanned 20 mm of the

sample through the beam at a constant speed of 0.5 mm/s. Tissue samples were exposed to either 0.5 or 1 J total incident energy over the 2 mm × 20 mm irradiated region (corresponding to either 125 or 250 J/cm^2 cummulative fluence) at either of the two laser wavelengths. Each exposure/wavelength condition was repeated on three individual samples, forming four groups of three tissue strips each.

Following irradiation, each sample was removed from the translation brace using stainless steel tweezers and held briefly beneath the surface of the LN_2 bath. A quartz EPR sample tube was immersed in the bath (filling it with LN_2), and the tissue strip was placed inside. The filled tube was then quickly transferred to a Dewar flask containing additional LN_2. Although any particulate ablation debris would have remained in the bath, the possible contamination of the tube by such debris was negligible. This was due to the fact that the bath volume (> 1 liter) was much larger than the tube fluid volume assayed by EPR spectroscopy.

Measurements of radical formation were made using a Varian E-line Century Series EPR spectrometer. EPR spectroscopy works on the principle that paramagnetic species such as free radicals will absorb microwave radiation when placed in a strong magnetic field. The specific microwave frequency and field strength required depend on the nature of the absorbing radical species. In this study the microwave frequency was fixed at 9.178 GHz, and absorption was measured as the magnetic field was scanned over the range 3,000–3,400 G. The other relevant spectroscopy conditions were a magnetic field modulation amplitude of 5 G and a microwave power of 10 mW. Tissue samples were kept at LN_2 temperature throughout the EPR assay.

Bacterial Cell Survival

Cell survival as a function of ultraviolet laser fluence at 193 nm and 213 nm was determined for E. coli BR339 (λ-lacZ, uvrB, lexA3). Cell suspensions were prepared following the methodology of Elespuru and White [12]. Cells were harvested during exponential growth (optical density at 600 nm ~ 0.4), concentrated 10 fold and stored at −55°C. Prior to irradiation, the frozen cells were thawed to room temperature and diluted 10 times with phosphate-buffered saline (PBS).

The laser fluence at the front surface of the cuvette was 1 mJ · cm^{-2} for 193 nm and 0.1 mJ · cm^{-2} for 213 nm. Irradiations were performed on 0.4 ml aliquots of cell suspension placed in a quartz cuvette (45 × 12 mm) having a 1 mm optical path length. Separate aliquots were exposed to a range of total ultraviolet doses by simply varying the number of incident laser pulses.

The cell suspensions were then transferred to small polystyrene tubes and held on ice. Samples including nonirradiated controls were diluted in PBS, plated for survivors on Luria agar, and incubated at least 18 hours at 37°C. To minimize the possibility for photoreactivation of potentially lethal DNA hits, room lighting was reduced to a level that allowed enough visibility for various manipulations. Plating for survivors was performed under red light.

Accurate determination of the fluence experienced by cells within the irradiation volume requires a correction for the transmission of radiation through that volume [13]. To this end, the front surface irradiance was corrected according to the method of Morowitz [14]. This method requires that the transmission of the irradiated volume be determined. However, the absorption of PBS limited the transmission of 193 nm radiation through the cell suspension to ~ 1%. The effect of medium transmission was examined in subsequent experiments in which cells were freshly harvested during exponential growth, washed, and resuspended in sterile, deionized water. The viability of water-suspended cells was found to be stable for > 1 hour in a separate test. Cell survivability in water suspensions was examined for three different cell densities exhibiting transmissions at 193 nm of 15, 34 and 85% (the 15% suspension was from frozen cell stock). Irradiation conditions for these experiments were identical to those for PBS media. The Morowitz correction was then applied to 193 nm irradiation of cells suspended in PBS by extrapolating the survival results of cells suspended in water.

RESULTS

Free Radical Formation

Composite EPR spectra obtained from the irradiated corneal tissue samples are shown in Figure 2. Each composite trace was calculated by averaging the three individual EPR spectra for a given irradiation condition to reduce noise in the individual spectra. The four curves in the figure are the composite spectra for the four different irradiation conditions. All four traces exhibit similar biphasic curve structure with a nearly iden-

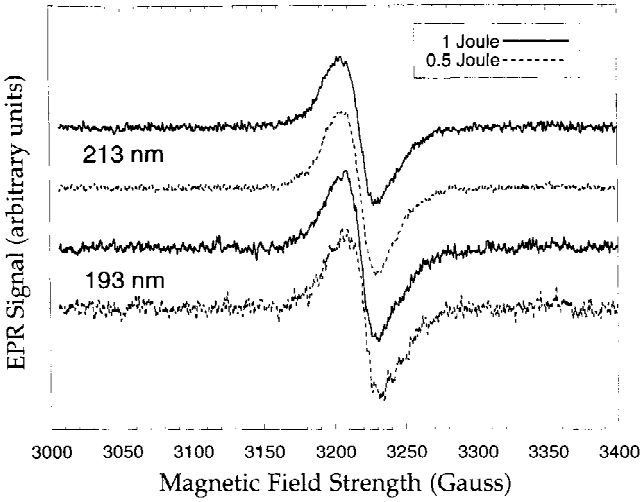

Fig. 2. The EPR spectra of ultraviolet laser irradiated corneal tissue. Each spectrum is the calculated composite of the individual spectra from three duplicate samples (see text). The phase-sensitive detection of EPR spectrometer produces a signal that is the derivative of the microwave absorption as a function of magnet field strength; hence the biphasic appearance of the spectra. The curves have been offset vertically for easier comparison. However, the vertical amplitudes of the signals are unmodified to allow direct comparison of signal magnitudes relative to the respective baselines.

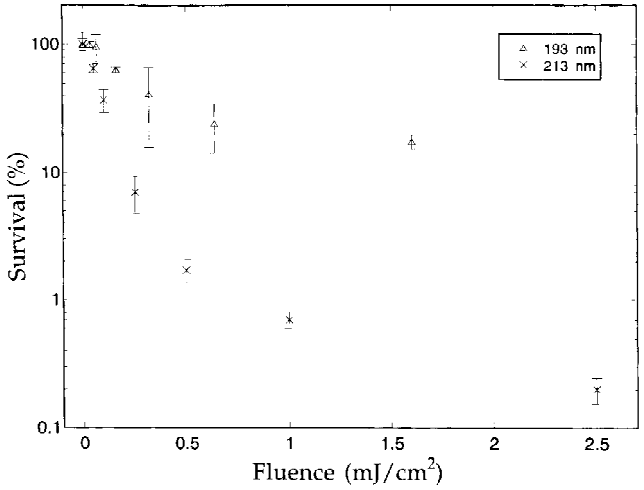

Fig. 3. Survival of BR339 cells suspended in PBS as a function of ultraviolet laser exposure. This population had been frozen at −55°C and was thawed immediately before UV exposure. Morowitz corrections to the incident irradiance were 0.03 and 0.5 for 193 and 213 nm, respectively.

tical linewidth (25.8 Gauss on average for the 193 nm data and 24.4 Gauss for the 213 nm spectra). No pronouced "shoulders" or other secondary structures exist in any of the traces.

Bacterial Cell Survival

As shown in Figure 3, the response of BR339 cells to 213 nm laser radiation is typical of many UV survival curves, with exponential killing through four orders of magnitude. However, the survival following 193 nm irradiation remains markedly high for fluences > 0.5 mJ · cm^{-2}. The survival data of water-buffered cells suspensions irradiated at 193 nm for three cell densities are depicted in Figure 4.

DISCUSSION

In our earliest EPR work involving corneal tissue [15], we consistently observed biphasic spectral features similar to those shown in Figure 2 for 193 nm irradiated tissue samples only (i.e., not for unirradiated controls). Thus the EPR results shown here are definitely due to a laser-tissue effect. Free radical production appears to be virtually identical for either ArF laser or frequency-quintupled Nd:YAG laser irradiation of cornea. The similarities in the shape of the EPR

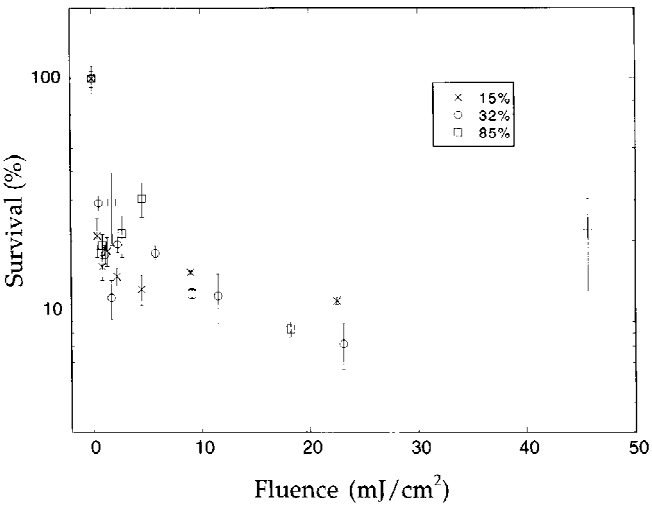

Fig. 4. Survival of BR339 cells suspended in water at three cell densities versus exposure to 193 nm laser radiation. Cell densities are denoted as their transmission in percent of 193 nm radiation over a 1 mm path (T_{water} = 100%). Cells in the 15% suspension were from frozen cell stock. Cells used for 32% and 85% suspensions were grown and harvested on the day of the experiment. The Morowitz correction factor for 193 nm irradiation of PBS-suspended cells (shown in Fig. 3) was extrapolated from these data.

spectra in Figure 2 suggest that the same types of radical species are formed at the two wavelengths. The amplitudes of the four EPR spectra in the figure are also quite comparable. This factor is directly proportional to the number of radical species present in the sample. Thus the quantum efficiency for radical formation also appears

roughly equivalent at 193 nm and 213 nm. Also notable is that no appreciable amplitude increase occurs between 0.5 J and 1.0 J cumulative exposures at either laser wavelength. This indicates a saturation in the radical accumulation for subablative irradiation of the tissue that is similar for both wavelengths.

In contrast, the cell survival experiments reveals significant differences between the response to 193 nm and 213 nm irradiation. The disparity in Figure 3 between the two wavelength results could be attributed to greater 193 nm shielding of the cells by the PBS solution. However, irradiation of aqueous cell suspensions by 193 nm results, shown in Figure 4, exclude this possibility. The water suspension data also demonstrates that survival at 193 nm is independent of cell density and illustrates the equivalency of frozen and freshly prepared cell stocks. This argues against the decreased 193 nm cytotoxicity being due to shielding of cells by either neighboring cells or wavelength specific photoproducts. Thus the dissimilarity of the survival data sets in Figure 3 implies that the targets leading to a lethal event is different for the two wavelengths. The 213 nm wavelength clearly has substantially greater cytotoxicity.

What implications do these results have for corneal sculpting procedures? The dominant chromophore in cornea for either of these laser wavelengths is the peptide bond linking adjacent amino acids in collagen [16]. It is therefore plausible that the free radicals observed are largely fragments of collagen polymers. The observed EPR spectra are in fact consistent with large organic radical species [10]. Also, laser-induced-fluorescence examination of ablation site and the ablation plume indicate that the water photodissociation radical OH^- is not abundant [17]. This sort of molecular damage, which would be repaired by the cornea as part of the healing response, appears to be an equivalent tissue insult for either laser device. However, if the differences seen here in bacterial cytotoxicity are applicable to mammalian cells, the corneal fibroblasts responsible for collagen repair [18] would be much more affected by 213 nm irradiation. This could help explain increased postoperative complications observed at the longer laser wavelength [9].

The results for these two experiments do not preclude the possibility of cellular-free radicals playing a role in the enhanced cytotoxicity of 213 nm laser radiation. As indicated above, this EPR study was largely an examination of laser effects on extracellular collagen. Cells contain many chromophores besides peptide bonds that strongly absorb in this wavelength region, e.g., nucleic acids, aromatic amino acids, etc. [16]. Therefore, the intracellular photochemistry brought about by these lasers is likely more complicated than that assayed in this corneal irradiation work and is an interesting area for future work.

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